RESTORATION OF ATP-INDUCED CONTRACTION OF "AGED" MITOCHONDRIA BY PHOSPHATIDYL INOSITOL

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Received April 18, 1963

Mitochondria "pre-aged" for several hours or days at 2° in 0.25 M sucrose are not able to contract, below pH 7.4, upon addition of ATP, bovine serum albumin (BSA) and Mg^{++} unless either a mitochondrial "contractile protein" or a second soluble protein fraction obtained from mitochondria is added (Vignais et al 1963). These protein fractions lose their activity after their lipid components are removed by extraction with chloroform-methanol. In such lipid fractions the ratio moles of acyl esters: moles of lipid phosphate was approximately 2.4, indicating the presence of mainly phospholipids. This paper is concerned with the identification of lipids capable of restoring the ATP-linked contraction of "pre-aged" mitochondria.

EXPERIMENTAL: Rat liver mitochondria were isolated according to Hogeboom (1955). Lipids from rat liver were extracted by chloroform-methanol (Folch et al 1957) and the phospholipids fractionated by chromatography (Hanahan et al 1957). Phosphatidyl inositol was prepared from beef liver (Faure et al 1958). Phosphatidyl serine and phosphatidyl ethanolamine separated during the preparation of phosphatidyl inositol were purified by chromatography on a DEAE cellulose column (Rouser 1962) followed by chromatography on silicic acid columns (Hanahan et al 1957). Also tested in this study were phosphatidic acid and phosphatidyl inositol

from wheat germ, phosphatidyl choline from egg yolk and cardiolipin from beef heart (gifts from Dr. M. Faure, Pasteur Institute, Paris), phosphatidyl choline and phosphatidyl inositol from beef heart (gifts from Dr. L. Wheeldon), phosphatidylglycerol from Staph. aureus, phosphatidyl ethanolamine and phosphatidyl serine from pig spleen (gifts from Dr. M. Gray, Lister Institute, London), sphingomyelin and phosphatidyl serine from beef brain (gifts from Dr. T. Preziosi). Other lipids were obtained from commercial sources. Swelling and contraction of mitochondria were followed spectrophotometrically at 520 mμ (Lehninger et al 1959) and/or by gravimetry (Price et al 1956). The different lipids used were added to the incubation mixture prior to swelling of the pre-aged liver mitochondria.

TABLE I

Effect of different lipid fractions from rat liver on ATP-induced contraction of mitochondria

Conditions: Mitochondria were pre-aged in 0.25 M sucrose at 2° for 2 days. Incubation mixture for measuring the swelling-contracting cycle: 0.125 M KCl, 0.02 M tris pH 7.4, oleate 10^{-5} M. A small inoculum of the mitochondrial suspension (380 μg protein) in 0.050 ml of 0.25 M sucrose was added to the medium supplemented or not with lipids. Swelling was allowed to proceed until the absorbancy dropped from 0.500 to 0.180, then the contraction was induced by addition of ATP (5 mM final conc.), BSA (2 mg/ml final conc.) followed after 5 min. by addition of $MgCl_2$ (3 mM final conc.). Temp. 25°.

Elution mixture $CHCl_3/CH_3OH$ v/v	Main phosphatide obtained and added at a final conc. of 1.5×10^{-4} M	Increment in Absorbancy at 520 mμ, 15 min after addition of ATP + BSA + Mg^{++} $\times 10^3$
-	none (control)	15
1/0	neutral lipids	0
9/1	cardiolipin	0
4/1	phosphatidyl ethanolamine phosphatidyl serine	22
3/2(1st peak)	phosphatidyl inositol	160
3/2(2nd peak)	phosphatidyl choline	13
0/1	sphingomyelin	0

RESULTS AND DISCUSSION: When lipids from rat liver were fractionated on a silicic acid column, the first fraction obtained by elution with $CHCl_3$-CH_3OH (3:2) was found to restore the ATP-induced contraction in pre-aged mitochondria (Table I). In agreement with the elution pattern given by Hanahan et al (1957), this fraction was shown to contain mainly phosphatidyl inositol with some traces of phosphatidyl choline (as determined by thin layer chromatography, Skipski et al 1962).

The following highly purified lipids or components isolated from different sources (see Experimental) were also tested on the contraction of pre-aged mitochondria, each at several concentrations between 10^{-10} M and 3.10^{-3} M: phosphatidic acid, phosphatidyl ethanolamine, lysophosphatidyl ethanolamine, phosphatidyl serine, phosphatidyl choline, phosphatidyl inositol, cardiolipin, phosphatidyl glycerol, sphingomyelin, phrenosine, acetylneuraminic acid, cholesterol, cholesterol esters, triolein, α and β glycerophosphate, linolenic, linoleic, arachidonic and palmitic acids, Vitamins A_1, D_3, K_1, E and Coenzyme Q_{10}. Among them, phosphatidyl inositol was the only component able to restore mitochondrial contraction to a significant extent at a low concentration; at 7.10^{-6} M phosphatidyl inositol from beef liver or from wheat germ brought about restoration of half maximum contraction. As shown in Table II, maximal contraction in the presence of phosphatidyl inositol required ATP, BSA and Mg^{++} and was inhibited by oligomycin.

Phosphatidyl choline from beef heart prepared according to Pangborn (1950) was able to restore half maximum contraction at 3.10^{-4} M; however after further purification on a silicic acid column (Hanahan et al., 1957) 3.10^{-4} M phosphatidyl choline could restore only 10% of the maximum contraction. Furthermore, egg lecithin was ineffective.

Phosphatidyl inositol restored ATP-induced contraction of mitochondria swollen spontaneously or in the presence of oleate (10^{-5} M), thyroxine (10^{-5} M), Ca^{++} (10^{-4} M) or inorganic phosphate (5.10^{-3} M).

Table II

Requirement for contraction of aged mitochondria

Conditions: Mitochondria were pre-aged in 0.25 M sucrose at 2° for one day. Incubation medium as in Table I except that ATP, BSA and Mg^{++} were added together. In expt. 1, 25 ml aliquots were taken for gravimetric measurements. In expt. 2 oligomycin (2 γ/ml) was added 5 min. before the addition of ATP. At the beginning of the swelling the absorbancy was 0.565 in expt. 1 and 0.580 in expt. 2. At the end of the swelling it was 0.180 in both cases. 839 μmoles of water were gained during the swelling phase (expt. 1). The last 2 columns report values obtained after 15 min. of the reversal phase.

Expt.	Phosphatidyl Inositol 8×10^{-5} M	Additions at the end of swelling	ΔA_{520} (15 min) $\times 10^3$	Extrusion of water (15 min) (μmoles)
1	-	ATP,BSA,Mg^{++}	- 5	- 40
	+	ATP,BSA,Mg^{++}	+267	+683
	+	ATP,Mg^{++}	+147	+527
	+	ATP,BSA	+ 22	+150
	+	BSA,Mg^{++}	+ 27	+180
2	-	ATP,BSA,Mg^{++}	+ 55	
	-	ATP,BSA,Mg^{++}, oligomycin	0	
	+	ATP,BSA,Mg^{++}	+308	
	+	ATP,BSA,Mg^{++}, oligomycin	+ 43	
	+	BSA,Mg^{++}	+ 80	
	+	BSA,Mg^{++}, oligomycin	+ 83	

No restoration occurred after swelling in an hypotonic medium or with reduced glutathione. Furthermore, phosphatidyl inositol was able to prevent the loss of dinitrophenol-stimulated ATPase activity and the unmasking of Mg^{++}-stimulated ATPase activity which occur during the swelling of mitochondria at 20°. This result supports the view that ATP-induced contraction of swollen mitochondria involves a part at least of the coupling mechanism of oxidative phosphorylation (Lehninger, 1962).

These findings may be related to the observations of Wojtczak and Lehninger (1961) and Wojtczak et al. (1963) on the formation of free fatty acids during the swelling and their incorporation into the phosphatidic

acid fraction of the membrane lipids during ATP-induced contraction. The latter authors have shown that both free fatty acids and α-glycerophosphate are essential for contraction under certain conditions. Thus two specific lipids found in the mitochondrial membrane are implicated in ATP-induced contraction of mitochondria under different conditions: phosphatidic acid or cardiolipin, and phosphatidyl inositol. Presumably phosphatidyl inositol is responsible for the restoration of mitochondrial contraction by protein fractions isolated from fresh mitochondria (Vignais et al., 1963).

It is noteworthy that a role of phosphatidyl inositol in the transport of cations across membranes has been suggested by work of Hokin and Hokin (1959), Ellis and Hawthorne (1962), and Garbus et al. (1963). Further studies on the specificity and enzymatic mechanisms on these lipid effects are in progress.

The authors thank Miss B. Ayers for expert technical assistance. This investigation was supported by grants from the National Institutes of Health, the National Science Foundation, the Nutrition Foundation, Inc., and the Whitehall Foundation.

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